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ORIGINAL ARTICLE

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Molecular cloning and expression of a novel klotho-related protein

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Abstract Klotho protein is a novel β-glucosidase-like protein produced predominantly in the kidney. The *klotho* mouse, which genetically lacks *klotho* gene expression, manifests various systemic phenotypes resembling aging. In the present study we succeeded in isolating a novel human protein structurally related to klotho protein. The protein possesses one β-glucosidase-like domain and is 42% identical with klotho protein at the amino acid level. Unlike klotho protein, it possesses neither a signal sequence nor a transmembrane domain, suggesting that it is a cytosolic protein, and thus was termed cytosolic β-glucosidase-like protein-1 (cBGL1). By Northern blot analysis cBGL1 mRNA was expressed most abundantly in the liver, followed by the small intestine, colon, spleen, and kidney. When *klotho* and *cBGL1* gene expression was examined in renal cell carcinoma tissues, both klotho and cBGL1 mRNA levels in tumors were lower than those in nontumor regions, suggesting that renal epithelial cells may lose *klotho* and *cBGL1* gene expression during the course of malignant transformation. In conclusion, we describe the primary structure and gene expression of a novel protein related to klotho protein.

Key words Klotho · β-Glucosidase · Gene expression · Kidney · Renal cell carcinoma

Abbreviations *CA:* Carbonic anhydrase · *cBGL1:* Cytosolic β-glucosidase-like protein-1 · *EST:* Expressed sequence tag · *G3PDH:* Glyceraldehyde-3-phosphate dehydrogenase · *LPH:* Lactase-phlorizin hydrolase · *RACE:* Rapid amplification of cDNA ends · *RCC:* Renal cell carcinoma · *RT-PCR:* Reverse transcription-PCR

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The nucleotide and deduced amino acid sequences reported in this paper have been submitted to the GenBank, EMBL, and DDBJ databases with the accession number AB017913

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Introduction

Klotho protein is a newly identified protein whose inborn deficiency in mice causes a broad spectrum of abnormalities such as arteriosclerosis, gonad and skin atrophy, pulmonary emphysema, and osteopenia, which are often seen in the aging process [1]. The *klotho* gene encodes secreted and membrane-bound forms of the protein, containing one and two β-glucosidase-like domains, respectively [2]. To date little is known about the molecular mechanism underlying the actions of klotho protein on various organs, and the regulation of *klotho* gene expression is understood only poorly, especially in humans. In the present study we aimed to identify novel human proteins structurally related to klotho protein and examine the primary structure and gene expression. Since the kidney is one of the organs most abundantly expressing

the *klotho* gene, we further investigated expression of both *klotho* and the novel genes in human renal cell carcinoma (RCC) tissues to examine their clinical significance in association with malignancy [3, 4].

Materials and methods

Molecular cloning of *cBGL1*

Using nucleotide and deduced amino acid sequences of human klotho cDNA [1] as probes, databases such as "dbest" in National Center for Biotechnology Information were searched by a basic local alignment search tool algorithm, especially with "tblastn" [5]. Three expressed sequence tag (EST) clones encoding novel human polypeptides highly homologous to klotho protein were obtained, which have accession numbers AA376807, H60622, and T83017. Clone AA376807 had been isolated from the small intestine, and clones H60622 and T83017 from the fetal liver and spleen. Each cDNA clones were successfully generated by reverse transcription-polymerase chain reaction (RT-PCR) using cDNAs made from mRNAs of human small intestine or fetal liver, which were obtained from Clontech. Thereafter several sets of RT-PCR were performed, and these EST clones were confirmed to be partial fragments of a single cDNA by use of the following primers: 5′-cttgtggcagctacactctgtgg-3′ (nucleotides 155–177, Fig. 1) and 5′-ccattgtttcggatgatcttggc-3′ (nt 1391–1369).

Rapid amplification of 5′- and 3′-cDNA ends

Clones containing the 5′ and 3′ untranslated regions of cytosolic β-glucosidase-like protein-1 (cBGL1) cDNA were generated by 5′- and 3′-rapid amplification of cDNA ends (RACE) using Marathon cDNA amplification kit with human fetal liver cDNA (Clontech) as previously described [6]. Gene-specific primers and nested gene-specific primers used were 5′-tatggtgatccactgcttgacacg-3′ (nt 489–466) and 5′-aagcggtaatgagtcaatccaagc-3′ (nt 227–204) for 5′-RACE, and 5′-agcgcctcttgatgacactcaacg-3′ (nt 1143–1166) and 5′-ccaagtaatatgccaagatcatcc-3′ (nt 1358–1381) for 3′-RACE. RACE products generated by nested PCR, approximately 0.3 kb for 5′-RACE and 0.8 kb for 3′-RACE in size, were subcloned into pGEM-T Easy Vector (Promega) and sequenced.

DNA sequencing

Nucleotide sequences were determined on both strands by Dye Terminator Cycle Sequencing Kit FS and 373B DNA sequencer (Applied Biosystems).

Northern blot analysis

Partial cDNA fragments corresponding to each of three EST clones were generated by RT-PCR (see above); for instance, primers 5′-cttgtggcagctacactctgtgg-3′ (nt 155–177) and 5′-tatggtgatccactgcttgacacg-3′ (nt 489–466) were used for EST clone AA376807 (Fig. 3). These cDNA fragments were labeled with [^{32}P]dCTP, and Northern blot analysis was performed as previously described [6]. Each lane contained 2 µg poly(A)$^+$ RNA (Human Multiple Tissue Northern Blot, Clontech). The blots were exposed against BAS-III imaging plate (Fuji) for 30 h. The blots were also hybridized with glyceraldehyde-3-phosphate dehydrogenase (G3PDH) cDNA probe (Clontech).

Human samples

To examine *klotho* and cBGL1 gene expression in the kidney total RNA was extracted using TRIzol reagent (Gibco BRL) from RCC tissues in kidneys of three patients who underwent nephrectomy (patients 1–3) at Kyoto University Hospital. Patient 1 (55-year-old man), patient 2 (74-year-old man), and patient 3 (67-year-old woman) had clear-cell type RCCs [3, 4] with tumor sizes of 35 mm (histological grade 2), 30 mm (grade 2), and 30 mm (grade 1) in diameter, respectively. Only patient 1 received interferon-α administration preoperatively, and no other chemotherapy or irradiation was carried out in the three patients. Tumor (T), lymph node (N), and metastasis (M) classifications were all T1N0M0. Nontumor region from subcapsular cortex, which was more than 4 cm away from each tumor region by pathological inspection, was also obtained and used as a source of RNA. The present study was conducted under informed consent and was approved by the ethics committee on human research of Kyoto University Graduate School of Medicine.

Reverse transcription-polymerase chain reaction

Of the total RNA extracted from human kidneys 5 µg was reverse-transcribed using oligo $(dT)_{15}$ primer and SuperScript II (Gibco BRL) at 42°C for 50 min in a total volume of 20 µl. PCR was performed in solutions containing 1 µl cDNA, 5 pmol primers, 200 µM dNTPs, 0.5 µl rTaq (Takara), and appropriate buffer. To verify equivalence of reverse-transcription reaction of each samples, four of the following six primers were mixed in a single tube: G3PDH sense, 5′-gaccacagtccatgccatcact-3′ (RT-PCR primer sets, Clontech); G3PDH antisense, 5′-tccaccaccctgttgctgtag-3′; klotho sense, 5′-gcatggtccctcatggatgg-3′ (nt 1377–1396) [1]; klotho antisense, 5′-tcctcttcttggtcacaacc-3′ (nt 1713–1694); cBGL1 sense, 5′-cttgtggcagctacactctgtgg-3′ (nt 155–177, Fig. 1); cBGL1 antisense, 5′-tatggtgatccactgcttgacacg-3′ (nt 489–466). The reaction profile was 94°C 30 s, (94°C 20 s, 62°C 30 s, 72°C 30 s) for 34 cycles, 72°C 10 min. Of the 50 µl PCR products 10 µl was electrophoresed in a 2% agarose gel and stained with ethidium bromide (Fig. 4). Non-tumor-region derived RNA from patient 2 was also treated similarly as other samples except that SuperScript II was omitted in reverse transcription reaction.

Results

Molecular cloning of *cBGL1*

Using nucleotide and deduced amino acid sequences of human klotho cDNA [1] as probes, we screened nucleotide sequence databases in National Center for Biotechnology Information. Three human EST clones were identified. The deduced amino acid sequences were 39–53% identical with that of klotho protein at amino acid positions 128–220, 333–368, and 426–505. We isolated partial cDNA fragments corresponding to these EST clones, and examined tissue distribution of their gene expression by Northern blot analysis. Their mRNA sizes and distributions were almost identical (one of the three is shown in Fig. 3), suggesting that these clones encode a single protein. By several sets of RT-PCR these EST clones were confirmed to be partial fragments of a single cDNA (data not shown).

To obtain the full-length cDNA, we performed 5′- and 3′-RACE, and finally succeed in determining the full-length cDNA sequence (Fig. 1). The largest open reading frame encoded a 469 amino acid protein with an estimated molecular mass of 53.7 kDa. This protein possessed a β-glucosidase-like domain highly homologous to klotho protein [7, 8]. Unlike klotho protein, it contained no hy-

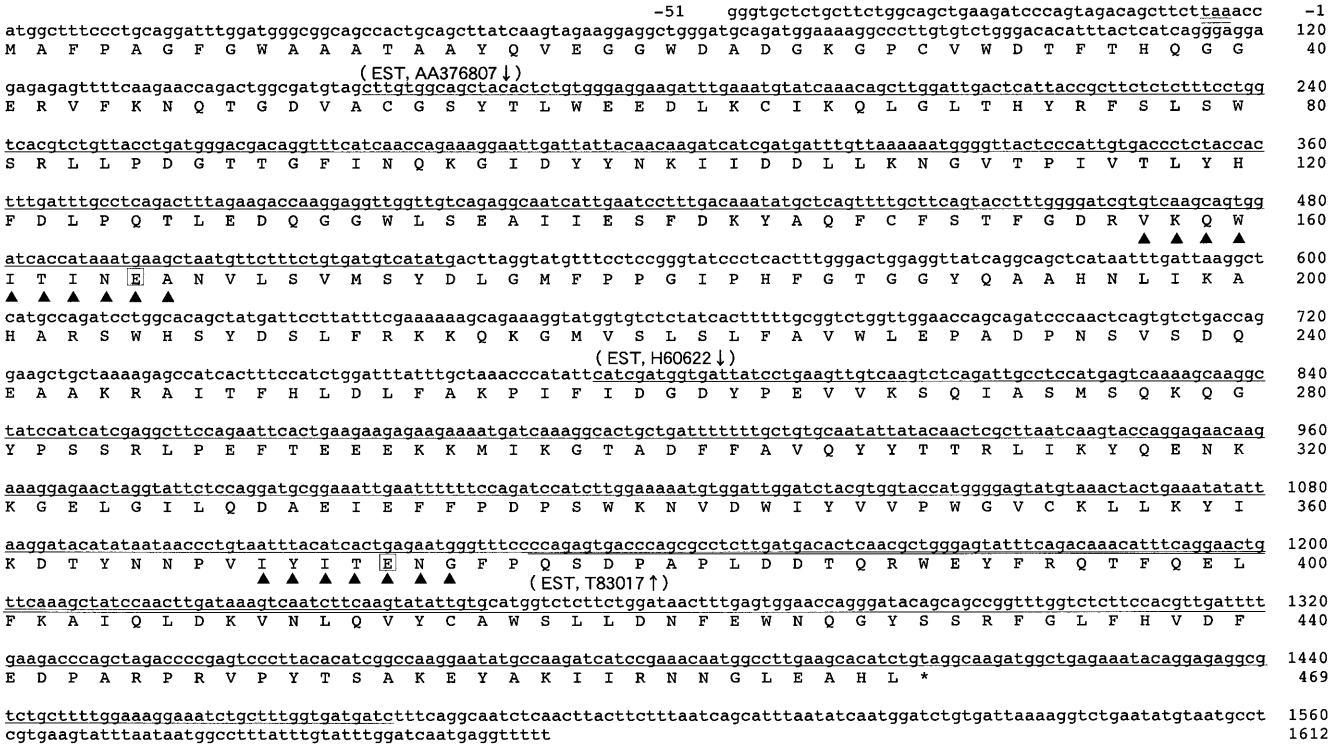

Fig. 1 Nucleotide and deduced amino acid sequences of human cytosolic β-glucosidase-like protein-1 (cBGL1) cDNA. *Underlined* cDNA fragments corresponding to three EST clones with accession numbers AA376807, H60622, and T83017. Nucleotides and amino acids are numbered sequentially from the translation initiation site. The translation initiation codon is preceded by an in-frame termination codon (*double underline*); *asterisk* termination codon; *triangles* conserved regions of β-glucosidase around two active site glutamic acid residues (*box*)

drophobic regions corresponding to signal sequence or transmembrane domain, suggesting its subcellular localization in the cytoplasm, and thus was termed cytosolic β-glucosidase-like protein-1. cBGL1 contained two conserved active site glutamic acid residues characteristic of β-glucosidase and fulfilled, although not completely, consensus sequences around the active sites: V-K-X-W-I-T-X-N-E-P at amino acid positions 157–166 and I-Y-I-T-E-N-G at positions 369–375 (Fig. 1) [7, 8]. These findings suggest that cBGL1 possesses β-glucosidase activity.

cBGL1, isolated in this study, possessed a single β-glucosidase-like domain. The membrane-bound form of klotho protein contains two repeats of β-glucosidase-like domains, KL1 and KL2, whereas the secreted form contains only one domain, KL1 [2]. To date it is not yet known whether klotho protein has β-glucosidase activity. cBGL1 was also homologous to lactase-phlorizin hydrolase (LPH), which is a membrane-bound protein containing four repeats of β-glucosidase-like domains, LPH1–LPH4 [9]. Amino acid sequence alignment of β-glucosidase-like domains of human klotho-related proteins and other proteins is shown in Fig. 2. When the amino acid sequences were compared, human cBGL1 was 42% and 32% identical with KL1 and KL2 of human klotho protein [1], and 41%, 47%, and 48% identical with LPH2, LPH3, and LPH4 of human LPH [9]. Furthermore, cBGL1 had 84% identity with *Cavia porcellus* (or guinea pig) cytosolic β-glucosidase [10], which can be the counterpart of cBGL1. cBGL1 also possessed 41% identity with *Arabidopsis thaliana* (a kind of plant) β-glucosidase homolog.

Tissue distribution of cBGL1

Northern blot analysis with cBGL1 cDNA probe revealed a 2.6-kb single transcript expressed most intensely in the liver, followed by the small intestine, colon, spleen, and kidney (Fig. 3).

Klotho and *cBGL1* gene expression in RCC

Klotho protein is a possible blood circulating factor and kidney seems to be one of the most important sources of klotho protein production [1, 2]. In murine kidney the *klotho* gene is expressed in the distal tubule [1]. However, renal proximal tubular epithelial cells from normal human kidney also express the *klotho* gene [11], and localization of *klotho* gene expression in human kidney is still only poorly understood. We investigated both *klotho* and *cBGL1* gene expression in clear cell type RCC tissues, which have been shown to originate from the proximal tubule (Fig. 4) [3, 4].

All nontumor regions expressed both klotho and cBGL1 mRNA at variable levels. PCR products for klo-

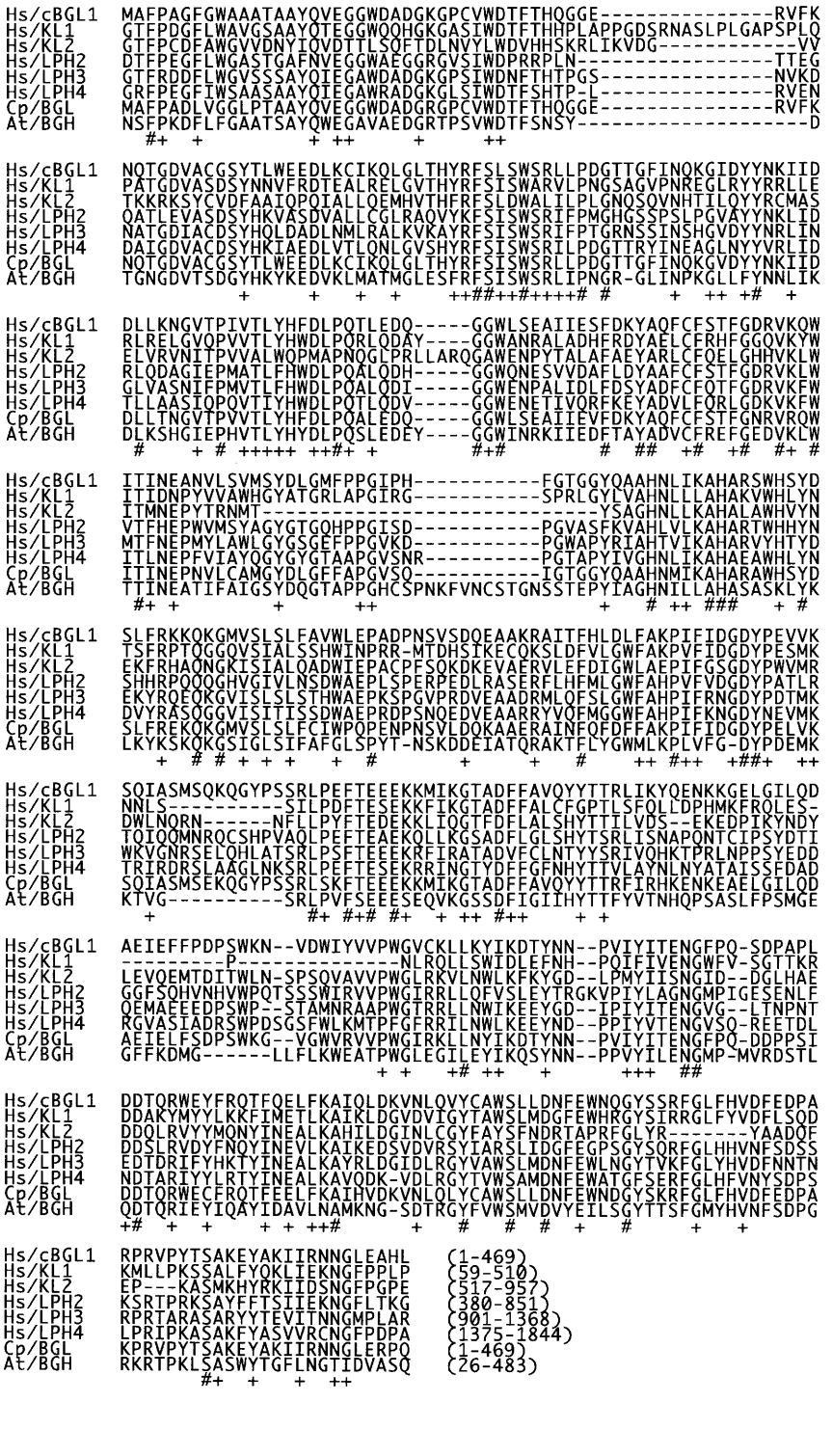

Fig. 2 Amino acid sequence alignment of β-glucosidase-like domains of human klotho-related proteins and other proteins. Sequences of Hs/KL1-2 were obtained from *Homo sapiens* klotho protein (GenBank accession number AB005142); Hs/LPH2-4 from *Homo sapiens* lactase-phlorizin hydrolase (SwissProt P09848); Cp/BGL from *Cavia porcellus* cytosolic β-glucosidase (GenBank CPU50545); At/BGH from *Arabidopsis thaliana* β-glucosidase homolog (GenBank AL078579). *Parentheses* Amino acid positions of each domain; # completely conserved amino acid residue; + homologous amino acid residue or one shared in seven of eight sequences

tho cDNA contained large and small bands, corresponding to secreted and membrane-bound forms, respectively [2]. In each lane the small band was much more abundant than the large band, suggesting that the membrane-bound form predominates. We found no correlations of mRNA levels with age, sex, renal function, or tumor size, although these analyses cannot be considered definitive due to the small numbers involved.

In each case the tumor region expressed lower levels of klotho and cBGL1 mRNA than did the nontumor region. The mRNA levels in tumor regions also varied between the cases. This may be associated with the differences in the clinical histories of the patients, the treatments before operations, or the cell heterogeneity in tumor samples: tumor regions consisted primarily of malignant cells, with some infiltration of lymphocytes, in-

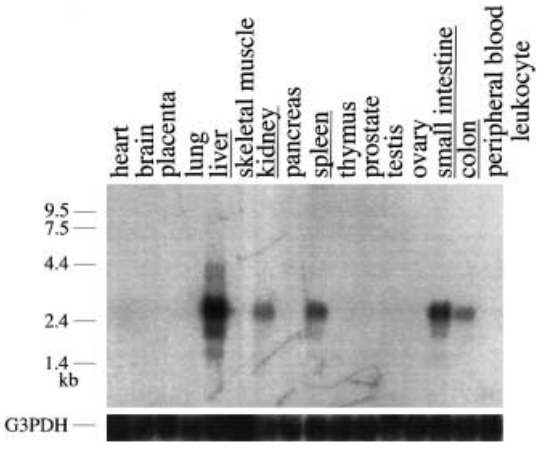

Fig. 3 Northern blot analysis of *cBGL1* gene expression in tissues. A cDNA fragment containing nucleotide positions 155–489 was labeled with [^{32}P]dCTP and used as a probe. The blots were rehybridized with G3PDH cDNA probe. *Underlined* Tissues with apparent *cBGL1* gene expression; *on the left* Positions of RNA size marker

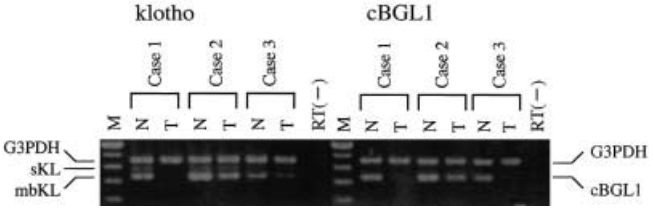

Fig. 4 *Klotho* and *cBGL1* gene expression in renal cell carcinomas. Gene expression of *klotho* and *cBGL1* was examined by RT-PCR in tumor regions (*T*) and nontumor regions (*N*). *M* Size marker (200, 300, 400, 500, and 600 bp); *RT(–)* without reverse transcription (see text). To verify that equivalent amounts of RNA were analyzed, PCR products for G3PDH were also amplified. Specificities of PCR products as membrane-bound (336 bp) and secreted (386 bp) forms of klotho protein, cBGL1 (335 bp), and G3PDH (452 bp) were confirmed by direct sequencing

vasion of tumor-feeding vessels, and necrotic tissues, but little involvement of stromal cells (data not shown).

Discussion

In the present study we succeeded in isolating a novel human klotho-related protein, cBGL1. cBGL1 possessed one β-glucosidase-like domain fulfilling consensus sequence characteristic of β-glucosidase. What are the physiological implications of cBGL1 being cytosolic as opposed to membrane-bound or secreted, as is the case with klotho protein and LPH? Phenotypes in the *klotho* mouse, caused by genetic lack of *klotho* gene expression, can be rescued by ectopic overexpression of the membrane-bound form of klotho cDNA, and the secreted form of klotho protein is also encoded by an alternatively spliced mRNA [1, 2]. These findings raise the possibility that klotho protein functions as an enzyme (whose substrates are provided by blood circulation), a secreted ligand, or a soluble receptor. If klotho protein and cBGL1 are enzymes (functional β-glucosidases), their functional implications in various subcellular localizations may be explained simply by the difference in their accessibility to different substrates. Even if they share common substrates, they might play distinct roles as follows.

As an example of a huge family of structurally related proteins with various subcellular localizations, carbonic anhydrases (CAs) have been extensively investigated [6, 12]. Some CAs are membrane bound while others are secreted, cytosolic, or mitochondrial. CA isozymes, which have only about 30–40% amino acid identity to one another, have carbon dioxide as a common substrate and catalyze reversible conversion into bicarbonate. They differ not only in their subcellular localizations but also in their tissue distributions, kinetic properties, and susceptibilities to inhibitors, and they have individual physiological roles. At least in the kidney the directions of the catalyzed reaction are believed to be opposite in extracellular and cytosolic CAs, and both of which are required for efficient bicarbonate reabsorption. It should be noted that receptor-type protein tyrosine phosphatase β has a CA-like domain without CA activity, and that its CA-like domain provides a binding site for a cell surface signal transducing molecule, contactin. Similarly, each of the klotho family proteins with characteristic β-glucosidase-like domains may have distinctive functions. The identification of target proteins or interacting proteins for each protein will facilitate our understanding concerning such issues.

As shown in Fig. 3, the *cBGL1* gene was expressed in various tissues. On the other hand, the human *klotho* gene has been shown to be expressed mainly in the kidney, placenta, prostate, and small intestine [2]; human LPH in the small intestine alone [9]; and guinea pig cytosolic β-glucosidase in the liver and kidney [10]. Thus each protein has unique tissue distribution, suggesting the differences in their functions.

As shown in Fig. 4, both klotho and cBGL1 mRNA levels in RCC tissues were lower than nontumor regions. These findings raise two possibilities: (a) renal epithelial cells lose *klotho* and *cBGL1* gene expression during the course of malignant transformation; (b) major source of *klotho* and *cBGL1* gene expression in human kidney may not be the proximal tubule, which is the origin of RCC, but may be the distal tubule, collecting duct, or glomerulus. When we examined *cBGL1* gene expression in hepatocellular carcinoma tissues, tumor regions similarly expressed lower levels of mRNA than nontumor regions (data not shown). Furthermore, β-glucosidase activity in colorectal adenocarcinoma is specifically lower among the various glycosidase activities than that in normal colonic mucosa, preferring the former possibility [13]. Until now only little is known on the precise anatomical distribution and function of either klotho protein or cBGL1, and nothing is known about the functional consequences of occurrence and disappearance of cBGL1 in normal and pathological tissues.

In conclusion, cBGL1 isolated in the present study is the third family molecule homologous to klotho protein in humans, which has only one β-glucosidase-like domain. The klotho protein and LPH protein are plasma membrane-bound proteins, while cBGL1 seems to be cytosolic. These three genes are commonly expressed in the small intestine, which may indicate a physiological role in nutrient metabolism. Comparison of structures and expressional regulation may facilitate further characterization of each protein. This study may bring new insights into our understanding of structure and expression of klotho family proteins.

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